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Mode of Action of Shigella Toxin: Effects on  
Ribosome Structure and Function

Annual Report

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## SUMMARY

The goals of this research contract are to examine the primary action of Shigella dysenteriae 1 toxin as an inhibitor of eukaryotic protein biosynthesis. Two major objectives of this investigation are designed to reveal Shiga toxin-induced changes in ribosome structure-function relationships. These objectives are 1) to explain, in biochemical terms, the manner by which Shiga toxin enzymatically inactivates mammalian ribosomes and 2) to define the steps of protein biosynthesis which are specifically inhibited by the toxin as a result of ribosome modification. A comparison between Shiga toxin and a similar plant-derived toxin is to be made in regard to ribosome inactivation. It is likely that information obtained from these studies will be of value in describing the role of Shiga toxin in establishment of intestinal infections by the toxin-producing Shigella species. In this contract period we have shown that Shiga toxin is an inhibitor of peptide elongation in reticulocyte protein synthesis. More exactly, we have demonstrated that Shiga toxin specifically inhibits aminoacyl-tRNA binding to ribosomes, a step catalyzed by protein synthesis elongation factor 2. Changes in ribosome structure as a result of toxin action was also investigated during the present contract period. It has been determined that Shiga toxin does not cause hydrolysis of ribosomal RNA to yield fragments larger than 10 nucleotides. Recent studies involving RNA sequencing indicate that the 3' terminal region of 5.8S ribosomal RNA remains intact following toxin inactivation of ribosomes. Examination of the 5' and 3' termini of the other ribosomal RNA species is in progress. Finally, comparative analysis of ribosomal proteins from control and toxin-treated ribosomes using the two-dimensional gel electrophoresis technique has resulted in identical patterns. It is concluded that: 1) Shiga toxin is an inhibitor of protein synthesis elongation and is without effect on the initiation process, 2) Shiga toxin does not hydrolyze ribosomal RNA into larger fragments and 3) the toxin does not result in modification of ribosomal proteins which would cause a major change in their mass or isoelectric points.

## FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH-78-23, Revised, 1978).

## BODY OF THE REPORT

### Statement of the Problem

1. Shiga toxin, as a documented inhibitor of mammalian protein biosynthesis, may specifically affect individual functional steps in the overall process.
2. The toxin, known to inactivate large ribosomal subunits, may interact with and modify a protein or RNA component of the ribosome.
3. Toxin-induced structural modification of the ribosome may be responsible for the change in ribosome protein biosynthetic activity.
4. Because the process of eukaryotic cytoplasmic protein biosynthesis is virtually identical in all mammalian cells and tissues, it is likely that information obtained from this study will be pertinent to Shiga toxin mode of action in intestinal tissues invaded by toxigenic Shigella.

### Technical Objectives

1. Describe, in biochemical terms, how purified Shiga toxin inhibits mammalian protein biosynthesis.
2. Identify the individual functional steps of protein synthesis which are inhibited by Shiga toxin.
3. Determine if the toxin-induced alteration in sub-cellular ribosome function is due to a structural modification of the ribosome.
4. Compare data from above with existing information on plant-derived toxins which resemble Shiga toxin mode of action at ribosome level.

### Background

It was established early on that a proteinaceous toxin is produced by the pathogen Shigella dysenteriae 1 (1). However, the relationship of the toxin and pathogenic properties of Shigella bacilli remains to be fully determined. During infection, Shigella penetrates the bowel epithelium and causes intestinal lesions (2). It has been suggested that a toxin produced by Shigella dysenteriae 1 is responsible for eliciting host responses to the pathogen (3,4). Host responses to Shigella include fluid production by ileal loops, in vitro, and diarrhea, in situ (4).

Moreover, a further understanding of Shiga action in the disease process would be aided by a series of investigations utilizing purified toxin. With partially purified toxin, several concepts pertinent to Shiga toxin function have become known. It appears that Shiga toxin is cytotoxic to several cell lines including HeLa human cervical carcinoma and WI-38 human fibroblasts (5-8). In addition, the relative activity of Shiga toxin in cell cultures is parallel to that in the rabbit ileum test system (6). From these data one might hypothesize that Shiga toxin is a non-selective agent which manifests its toxic properties on a wide array of cell and tissue types. However, recent

reports indicate that toxin from S. Shigae exhibits high-affinity binding to a limited number of cell types (8,9). This strongly suggest that the toxin resembles other well-known microbial-derived toxins (i.e., diphtheria toxin, cholera toxin and Pseudomonas exotoxin A) which bind with high affinity ( $K_d = 10^{-10}$  M) to receptors on cells (10).

All of the above mentioned toxins appear to be potent inhibitors of protein biosynthesis in eukaryotic cells. It is generally accepted that clinical symptoms associated with these toxin-producing bacteria are, in part, a result of their effect on protein synthesis. To go one step further, it has been suggested that Shiga toxin also elicits different physiological responses in the host target by virtue of its ability to efficiently inhibit protein by biosynthesis (11). Indeed, there is ample evidence indicating that Shiga toxin has, as its primary action, the inhibition of protein biosynthesis in whole animals (12) cell cultures (8,11) and cell-free lysates (11,14). It seems clear that ribosomes are the primary target of Shiga toxin and more recent results indicate that the large ribosomal subunit of eukaryotic cells are specifically affected (15). At this time, virtually nothing is known about the ribosomal component which is modified by Shiga toxin.

It is conceivable that such information would be used to advantage in design of a mode of therapy for Shiga toxin based on information regarding the ribosomal substrate. In addition, these studies should lead to implementation of a new molecular assay for Shiga toxin which would have great sensitivity and specificity.

Shiga toxin has been purified to apparent homogeneity from S. shigae (9,16) and S.dysenteriae 1 (17,18). The holotoxin from both sources has an approximate molecular weight of  $M_r = 70,000$  (9,18). Structural analysis of S. shigae toxin (9) indicates its similarity to cholera toxin; Shiga toxin has a single larger peptide of  $M_r = 30,000$  and multiple copies of a smaller peptide ( $M_r = 5,000$ ). It is also apparent that Shiga toxin resembles other multicomponent proteinaceous toxins (19) by having catalytic toxicity and cell binding specificity functions located on distinct peptides. That is, the larger peptide (subunit "A") is catalytic while smaller peptides (subunit "B"), appear to infer binding specificity properties of the holotoxin.

#### Approach to the Problem

##### a. General Information

It seems very possible that Shiga toxin could share many of the features of the plant-derived phytolectin toxin as an inhibitor of protein biosynthesis (24). Our research plan is designed to examine the action of Shiga toxin in the test systems we have successfully employed in the study of plant toxin mode of action. These investigations were designed, in part, because the Principal Investigator has had extensive experience in preparation and use of protein synthesis assay systems which would be useful in carrying out the Shiga toxin study (22-25).

The primary objective of this project is to describe, in biochemical terms, how Shiga toxin inhibits protein biosynthesis in mammalian cells.

To achieve this goal, two aspects of protein synthesis will be examined in reticulocyte (rabbit) cell-free protein synthesis systems. First, it will be determined what steps of protein synthesis (i.e., initiation or elongation) are affected by Shiga toxin. Second, we will determine how Shiga toxin inactivates mammalian ribosomes. More exactly, as Shiga toxin is known to specifically inactivate the large ribosomal subunit, a goal of this research plan is to determine how Shiga toxin modifies the structural integrity of the ribosome. Components of the large ribosomal subunit including its 47 proteins will be monitored for changes resulting from Shiga toxin action. The following is an abbreviated description of methodology to be used in the study of Shiga toxin mode of action as an inhibitor of protein biosynthesis. Abbreviations appearing include:

rRNA - ribosomal ribonucleic acid  
rprotein - ribosomal protein  
tRNA - transfer ribonucleic acid  
mRNA - messenger ribonucleic acid  
EF 1 - elongation factor 1 which is the aminoacyl-transfer RNA binding factor  
EF 2 - elongation factor 2 which is the translocase factor  
eIF - eukaryotic initiation factor  
Met-tRNA<sub>f</sub> - initiator methionyl-tRNA  
GTP - guanosine triphosphate  
DTT - dithiothreitol, a reducing agent  
polysome - a mRNA molecule with 3+ ribosomes attached  
80S ribosome - ribosome comprised of 60S and 40S subunits  
60S, 40S ribosome - large and small ribosomal subunits, respectively  
TCA - trichloroacetic acid

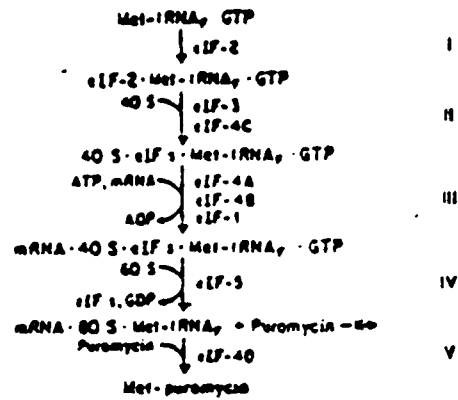
b. Initiation of protein synthesis

The following section is a description of refined initiation assays which are included in the research plan. It should be emphasized that we intend to examine, in detail, only those reactions affected by toxin as indicated by combined data from lysate and partially fractionated globin protein biosynthesis systems.

Initiation of protein synthesis consists of a series of reactions involving at least 3 initiation factor proteins, methionyl-tRNA<sub>f</sub>, GTP, ATP, mRNA and ribosomal subunits. These reactions appear to occur in a defined sequence as shown (26). Our plan is to study the effect of toxin on formation of initiation complexes in an unfractionated reticulocyte lysate. Rationale for the assay is that exogenously added, laboratory-synthesized [<sup>35</sup>S]Met-tRNA<sub>f</sub> will take part in initiation following the "Scheme A" (Page 9). However, should toxin block one or more of these steps, the [<sup>35</sup>S]Met-tRNA<sub>f</sub> will accumulate in an intermediate complex. Each of these complexes can be detected and identified by either separation on sucrose gradients following centrifugation, or by collection on nitrocellulose filters.



### Scheme A



### c. Elongation of Protein Synthesis

The three steps involved in peptide elongation are 1) binding of aminoacyl-tRNA to the ribosome, 2) formation of a peptide bond between amino acids in the growing protein catalyzed by peptidyl transferase and 3) translocation of peptidyl-tRNA from one site to another site on the same ribosome. All of these steps will be monitored in the absence and presence of Shiga toxin as described below. Although it seems inappropriate to give a complete description of the elongation process in this report, it should be pointed out that each of these individual steps in elongation may be monitored in one or more ways by utilizing radioactivity probes in each of the different components that take place in the reactions. During our experience with these assays, we have been able to describe the site of action of several rather specific inhibitors of eukaryotic protein synthesis (22-24).

The assay systems measure: 1) binding of aminoacyl-tRNA to the ribosome, 2) translocation of aminoacyl-tRNA on ribosomes, 3) Ef-2•GDP•ribosome complex formation, 4) EF-1 and EF-2 GTPase activity and 5) peptidyl transferase activity.

### d. Ribosomal Proteins

All of the 48 r-proteins found in 60S ribosomal subunits are separable by two-dimensional gel electrophoresis (26-28). This procedure will be used for our examination of the 60S subunit acidic and basic r-proteins isolated from untreated and toxin-inactivated 60S subunits. Proteins extracted from 60S particles will be separated in the first dimension by their electrophoretic mobility at pH 8.6 on a cylindrical 8% acrylamide gel. The second dimension separation is a function of size of r-proteins and is carried out at pH 4.2 in an 18% acrylamide slab gel (29). Variations in this general description include acrylamide concentration, pH, voltage, time of electrophoresis, and ratio of acrylamide to bis-acrylamide. These variations are considered important because of the possibility that an altered protein from toxin-treated ribosomes could assume a new co-migration pattern with any of the other r-proteins.

### e. Ribosomal RNAs

Although this section was not included in the initial research plan, we plan to examine the rRNAs of toxin-treated ribosomes to fully round-out our examination of toxin action at the ribosomal level. Three rRNA species located in the 60S ribosome are 5S, 5.8S and 28S rRNAs.

Examination of these rRNAs is accomplished in two parts. First, the size of rRNAs are analyzed on polyacrylamide gels. This will allow us to detect an enzymatic hydrolysis of the rRNAs which would yield RNA fragments of 10 nucleotides or larger. Second, the individual RNA species are studied for modification at their termini by the modern technique of RNA sequencing. In the latter case, each rRNA type is radioactive labeled with [ $^{32}\text{P}$ ], chemically hydrolyzed with nucleotide-specific reactions and fragments separated by gel electrophoresis. Using this technique one can determine the exact nucleotide sequence at the ends of each RNA.

## Results

### Summary of Time-Effort and Research Progress

An overview of the initial two-year contract period is presented in Tables 1 and 2. This is designed as a conceptual aid while a more detailed description of the results obtained are located in the following sections "a" through "f".

TABLE 1 SUMMARY: RESEARCH TIME-EFFORT ON ORIGINAL STATED OBJECTIVES

A./Shiga-Toxin Effect on Eukaryotic Protein Synthesis	B./ Shiga-Toxin Effect on Ribosome Structure	Contract Period
<p>Initiation<sup>1</sup></p> <p>↑ ↓</p> <p>Peptidyl-<sup>1</sup> Transferase</p> <p>↑ ↓</p> <p>EF-<sup>2</sup></p> <p>↑ ↓</p> <p>EF-<sup>2</sup></p>		Nov: 1982 (start of contract)
	<p>basic<sup>3</sup> r-proteins</p> <p>↑ ↓</p> <p>rRNA<sup>4</sup></p>	Mar. 1983
		July 1983
		Nov. 1983
	<p>acidic<sup>3</sup> r-proteins</p> <p>↑ ↓</p>	Mar. 1984
	<p>X-linking<sup>5</sup> Shiga toxin to ribosomes</p> <p>↑ ↓</p>	July 1984
(Data above contained in this report)		Nov.1984

TABLE 1 Footnotes:

The following commentary is a statement related to rate of progress.

PART A:

1. These data were obtained rapidly as the study was performed in reticulocyte lysate preparations which did not require purification of protein synthesis enzymes.
2. In contrast to the studies above (part A1), extra time was required to purify elongation factor 1(EF-1) from rabbit reticulocytes. The elongation factors EF-1 and EF-2 are labile proteins which have caused moderate difficulty in this part of the project. Examination of EF-2 dependent reactions underway at this time indicate Shiga toxin does not inhibit this step in protein synthesis. Acquisition of a HPLC unit through our recently approved (fiscal year 1985) DOD-University Research Instrumentation Program application will afford a more efficient means of dealing with purification of EF-2 protein.

PART B:

3. Examination of the numerous basic (pI=10) ribosomal proteins (r-proteins) by 2-D PAGE has gone smoothly. However, the few acidic r-proteins have yet to be separated.
4. A close look at 5S, 5.8S and 28S rRNAs from toxin-treated ribosomes went smoothly until we started RNA sequencing of the 5' and 3' ends which has been time consuming due the technical difficulties involved. We remain convinced that the extra time required to obtain these data is very much worthwhile and is an integral part of the methodical approach being taken.
5. Crosslinking of toxin to ribosomes was scheduled as the last of the projects to be undertaken. We have established that solid-state iodination and reaction of Shiga toxin with these ligands does not reduce biological activity of the toxin.

TABLE 2 SUMMARY: PROGRESS TOWARDS COMPLETION OF ORIGINAL RESEARCH GOALS

		Percent completed as of <u>06/01/84</u>	Results and conclusions	Data presentation at <u>annual ASBC</u> <u>meetings</u>	Data presentation in <u>journal form</u> <sup>3</sup>
A) <u>STEPS OF PROTEIN SYNTHESIS</u> <u>AFFECTED BY SHIGA-TOXIN</u>					
1.)	Initiation	100	No inhibition <sup>2</sup>	06/1983	M2 <sup>4</sup>
2.)	Peptidyl transferase	100	No inhibition <sup>2</sup>	06/1983	M2 <sup>4</sup>
3.)	Elongation factor 1 reactions	100	Strong inhibition	06/1984	M2
4.)	Elongation factor 2 reactions	50 <sup>1</sup>	No inhibition	04/1985	M2
B) <u>ALTERATION IN RIBOSOME</u> <u>STRUCTURE CAUSED BY</u> <u>SHIGA-TOXIN</u>					
1.)	Direct analysis of r-proteins (2-D PAGE)	100	No detectable <sup>5</sup> changes	04/1985	M2
2.)	Direct analysis of r-RNA <sub>6</sub>				
a)	rRNA hydrolysis into larger fragments (>10NT)	100	No fragments <sup>6</sup> generated	06/1983	M1
b)	3' exonuclease activity	40	None detected <sup>7</sup>	04/1985	M3
c)	5' exonuclease activity	50	To be completed	04/1985	M3
d)	demethylation activity (T1 di- gest mapping)	0	To be completed	04/1985	M3
e)	Non-selective endonuclease activity	100	None detected <sup>6,8</sup>	06/1983	M1
3.)	Photoaffinity X-link- ing of S1 to ribosome	15	To be completed <sup>9</sup>	06/1986	M3

TABLE 2 Footnotes:

PART A:

1. Purified EF-2 protein required for this study, from rabbit reticulocytes.
2. Partial inhibition was observed in the last step of initiation which requires 60S ribosomal subunits.
3. Submission to journals: M(manuscript) 1, Biochem. J. (Sept. 1984); M2, J. Biol. Chem. (Oct. 1984); M3, J. Biol. Chem.
4. Negative results obtained required coupling of these data, obtained early in the project, with positive data on EF-1 reactions (part A3), completed only recently.

PART B:

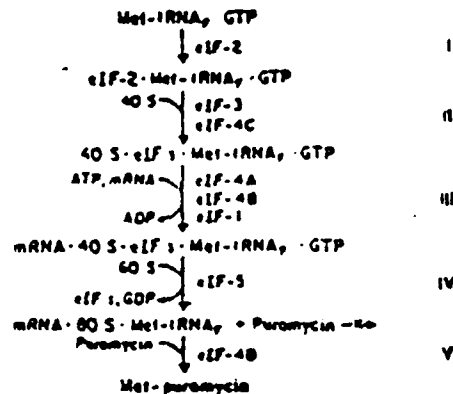
5. Separation of basic r-proteins based on mass and net charge at pH 8.6, 9.6 or 10.6.
6. Includes comparison of Shiga toxin data with those of alpha sarcin toxin (a known ribosome inhibitor with RNase activity) serving as a positive control.
7. Based on examination of 5S and 5.8S rRNA; 28S rRNA is presently being examined.
8. Includes examination of rRNA from Shiga toxin-inactivated ribosomes and of isolated "free" rRNA species exposed directly to toxin.
9. Recently established that reaction of photoaffinity ligand to Shiga toxin does not alter biological activity of toxin (in dark) as and inhibitor of protein synthesis.

## RESULTS

### a. Initiation of Protein Synthesis: Shiga toxin does not inhibit initiation of mammalian protein biosynthesis.

A detailed study was performed to determine if Shiga toxin affected initiation of eukaryotic protein biosynthesis. Results from this series of experiments were conclusive; Shiga toxin did not interfere with formation of intermediate complexes or reactions presented in scheme A.

Scheme A



These studies were conducted in either reticulocyte lysates with endogenous mRNA or in assay mixtures containing partially purified initiation factors, ribosomes, artificial mRNA and initiator [<sup>35</sup>S]methionyl-tRNA. Most of these components were prepared in Dr. Obrig's laboratory using established procedures. In the cause of brevity, results from one such experiment is presented (Table 3). The basis of this assay is formation of the final complex of initiation resulting from step no. 4 (above) and its reaction with puromycin to yield [<sup>35</sup>S]met-puromycin as indicated in step no. 5. Shiga toxin exhibited only a marginal effect on this assay, indicating the toxin did not inhibit steps numbered 1 through 5, i.e., the entire peptide initiation process.

## FEDERATION PROCEEDINGS

(1983) 42:1808.

SHIGA TOXIN DOES NOT AFFECT INITIATION OF PROTEIN SYNTHESIS.  
T.G. Obrig\*, T.P. Moran\* and J.E. Browne\* (SPON: J.L. Glenn).  
Albany Med. Col. Albany, NY 12208 and Walter Reed Army Inst.  
Res., Washington, DC, 20012.

Shiga toxin, the protein toxin of *Shigella dysenteriae* 1 has been shown to inhibit eukaryotic protein synthesis catalytically. We have now examined toxin action for its effect on initiation of protein synthesis. Toxin, purified to homogeneity from *S. dysenteriae* 1, strain 38180, was activated with TPCK-crypsin followed by phenylmethylsulfonyl fluoride, urea and dithiothreitol. A Shiga toxin concentration of 1 µg/ml which completely inhibits protein synthesis in reticulocyte lysate was utilized. In the presence of ribosomal salt wash fraction, formation of the ternary initiation complex [<sup>35</sup>S]Met-tRNA<sub>f</sub>-eIF-2-GTP was not affected by the toxin. Further, when sucrose gradient-isolated ribosomes were added, codon-directed binding of [<sup>35</sup>S]Met-tRNA<sub>f</sub> to the 40S ribosome was not inhibited by toxin. Toxin effects on protein synthesis initiation in complete reticulocyte lysates were monitored in the presence [<sup>35</sup>S]Met-tRNA<sub>f</sub>. Accumulation of the initiation complex [<sup>35</sup>S]Met-tRNA<sub>f</sub>-40S ribosomal subunit-mRNA, promoted in response to NaF and 8,γ-methylene GTP, was not inhibited in the presence of Shiga toxin. Finally, [<sup>35</sup>S]Met-tRNA<sub>f</sub>-60S ribosome-mRNA complex formed in Shiga toxin-treated lysates was fully capable of reacting with puromycin to yield [<sup>35</sup>S] methionyl-puromycin. We conclude that Shiga toxin inhibits protein synthesis without affecting the initiation process.

Table 3

[<sup>35</sup>S]Methionylpuromycin formation in unfractionated reticulocyte lysate

	<u>[<sup>35</sup>S], cpm</u>
minus lysate	315
complete, 4°C	265
complete, 37°C	4,315
complete, 37°C, Shiga toxin	3,885
complete, 37°C, HCR*	560

\* HCR is hemin control repressor, a protein kinase activated in lysates incubated at 37°C without hemin. HCR phosphorylates (inactivates) initiation factor eIF · 2. HCR was added to the assay, where indicated, at zero time as a positive control.

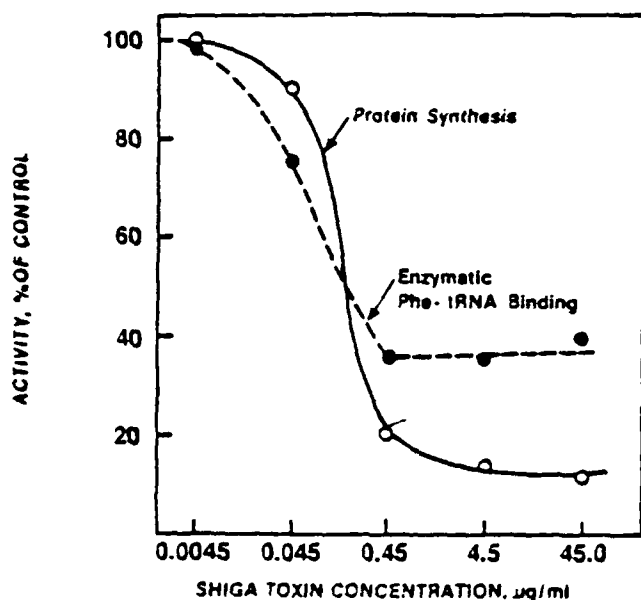


Fig. 1. Effect of Shiga toxin on total protein synthesis and phe-tRNA binding to reticulocyte ribosomes.

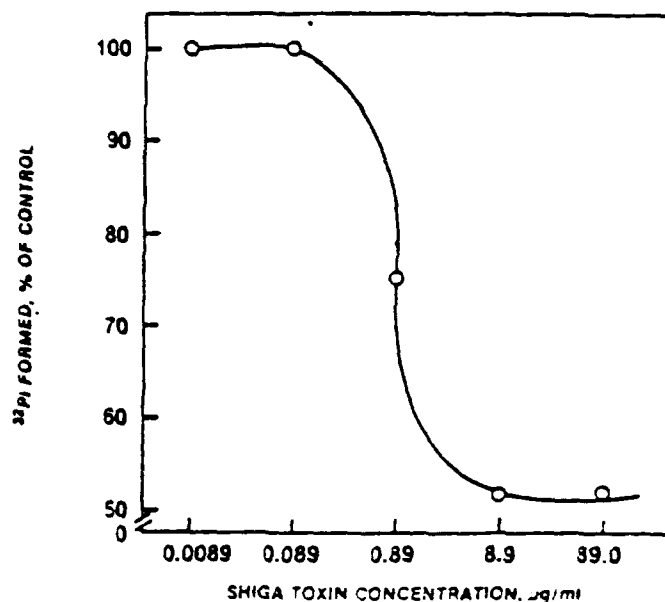


Fig. 2. Effect of Shiga toxin on EFl-dependent GTPase activity on reticulocyte ribosomes.



b. Elongation of protein synthesis: Shiga toxin specifically inhibits aminoacyl-tRNA binding to ribosomes.

These series of experiments measured elongation factor-1 (EF1)-dependent binding of [ $^3$ H]phe-tRNA to isolated reticulocyte ribosomes in the presence or absence of Shiga toxin. The phe-tRNA binding was GTP and poly(U) dependent and was catalyzed with purified eucaryotic EF1 protein. The data indicate Shiga toxin has a clear effect on this reaction (Fig. 1). Interestingly, the potency of Shiga toxin against phe-tRNA binding is virtually identical to its relative inhibitory activity for overall protein synthesis as measured by [ $^3$ H]leucine incorporation into globin protein in unfractinated reticulocyte lysates (Fig. 1).

Other data support the concept that the primary action of Shiga toxin is on EF1-catalyzed reactions which require intact ribosomes. The GTPase activity associated with EF1 protein was also inhibited by Shiga toxin in a dose-dependent manner (Fig. 2). These effects of Shiga toxin result from its action on 60S ribosomal subunits rather than on EF1 directly as excess EF1 protein could not overcome the inhibition.

FEDERATION PROCEEDINGS

(1984) 43:1953.

INHIBITION BY SHIGA TOXIN OF ELONGATION FACTOR 1-DEPENDENT REACTIONS OF PROTEIN SYNTHESIS. Don C. Orig<sup>a</sup>, J. Edward Brown, and Tim P. Moran<sup>b</sup>. Albany Med. Coll., Albany, NY 12208, and Walter Reed Army Inst. Res., Washington, DC 20307

Shiga toxin, a protein toxin of *Shigella dysenteriae* 1 previously shown to inhibit enzymatically eucaryotic protein synthesis, has now been examined for a direct effect on elongation factor 1 (EF-1)-dependent reactions. Purified toxin from *S. dysenteriae* 1 strain 3818-0, was activated using TPAK-tryptsin, followed by phenylmethylsulfonyl fluoride, 10 M urea and 10 mM dithiothreitol. Shiga toxin inhibited [ $^3$ H]-Phe-tRNA binding to ribosomes when tested in reaction mixtures containing poly(U), purified EF-1, and 0.5 M KCl-washed reticulocyte ribosomes. Binding was inhibited 24% and 68% by 0.045 and 0.45  $\mu$ g toxin/ml, respectively. Similar levels of inhibition were observed with  $\alpha$ -sarcosine, whereas no inhibition was detected with diphtheria toxin. Inhibition by Shiga toxin was not affected by either rate-limiting or saturating amounts of EF-1 protein. Concentrations of toxin which inhibited EF-1-dependent Phe-tRNA binding caused similar reductions of total protein synthesis in reticulocyte lysate reaction mixtures. Non-enzymatic binding of Phe-tRNA to ribosomes was not affected. EF-1-dependent enzymatic GTPase was inhibited by Shiga toxin in a dose-dependent manner in mixtures containing EF-1, Phe-tRNA, ribosomes and [ $\gamma$ - $^{32}$ P] GTP. The degree of inhibition was equal to that observed with phytolectin (PAP) or  $\alpha$ -sarcosine. We conclude that Shiga toxin has a direct inhibitory effect on aminoacyl-tRNA binding reactions of peptide elongation.

c. Elongation of Protein Synthesis: Shiga toxin appears not to inhibit elongation factor-2 reactions

The most recent data from our laboratory indicate that Shiga toxin has no effect on EF-2-dependent reactions. An identical concentration of toxin maximally inhibits total protein synthesis in reticulocyte lysates. The EF-2 dependent assay was a measure of GTPase activity in a system containing buffer, potassium and magnesium salts, and [ $^{32}$ P]-GTP.

d. Shiga toxin does not modify ribosomal proteins

Numerous attempts were made to reveal a Shiga toxin-related change in one or more of the 60S ribosomal subunit proteins. These proteins were isolated from either toxin-treated or control ribosomes and analyzed by 2-D PAGE. Separation in the first dimension was by net charge, and in the second dimension by protein mass. Results of typical r-protein patterns are presented in Figure 3. Although we conclude that no

toxin-related changes occur in these parameters, it is possible that Shiga toxin could modify the r-proteins in a way which would not be detectable by this 2-D PAGE procedure.

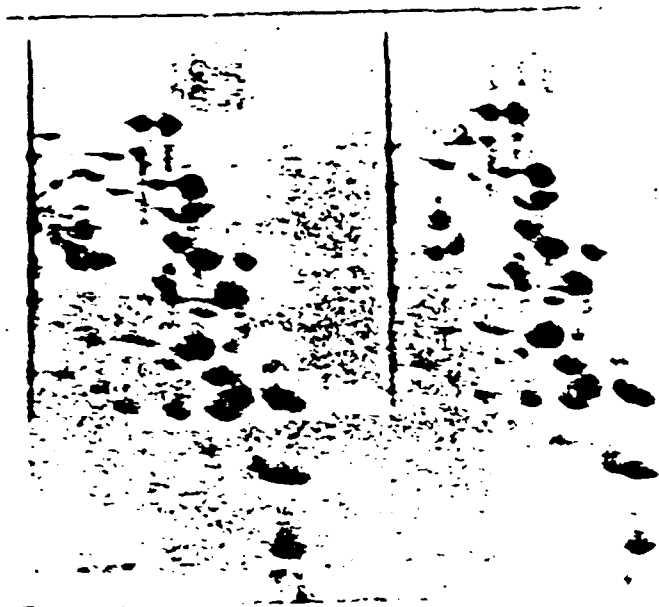


Fig. 3. Effect of Shiga toxin on r-proteins of 60S ribosomal subunits. Reticulocyte polysomes were incubated with (right) or without (left) toxin and 60S subunits isolated. The r-proteins were extracted and analyzed by 2-D PAGE.

e. Ribosomal RNAs: Shiga toxin does not exhibit RNase activity similar to alpha-sarcin

FEDERATION PROCEEDINGS

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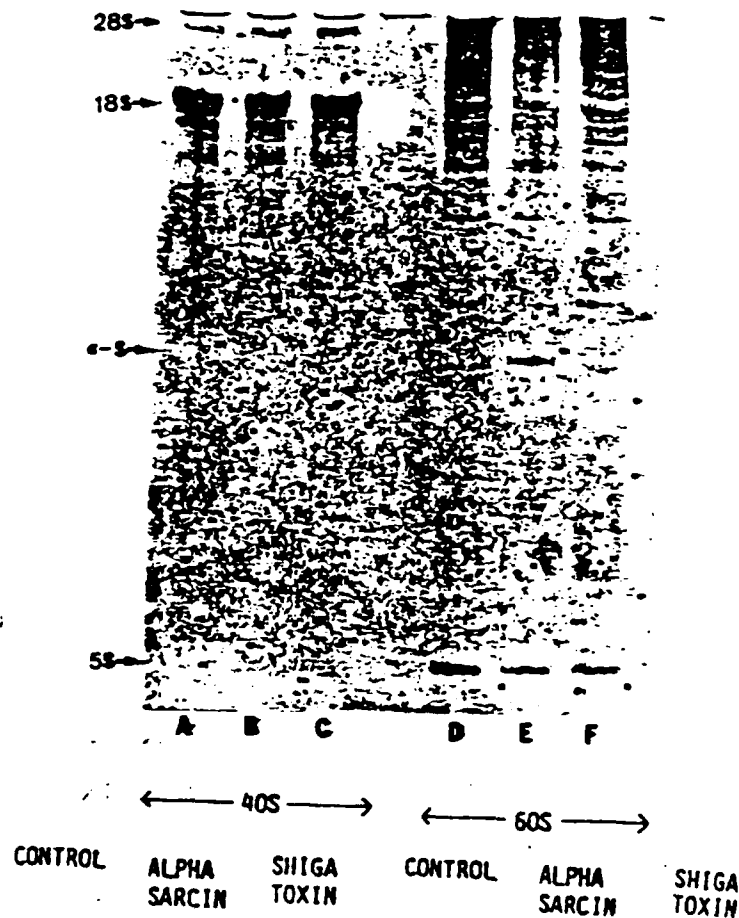
ON THE MECHANISM OF ACTION OF SHIGA TOXIN IN THE INHIBITION OF EUKARYOTIC PROTEIN SYNTHESIS. T.P. Moran<sup>a</sup>, T.C. Obrig<sup>a</sup> and J.E. Brown<sup>a</sup> (SPON: J.L. Glenn). Albany Med. College, Albany, NY, 12208 and Walter Reed Army Inst. Res., Washington, DC, 20012.

We have observed that Shiga toxin appears to affect eukaryotic protein synthesis by inhibition of the aminoacyl-tRNA binding step of peptide elongation in a manner similar to that of  $\alpha$ -sarcin. Since the mechanism of  $\alpha$ -sarcin involves hydrolysis of 28S rRNA in 60S ribosomal subunits, the action of Shiga toxin on 28S rRNA was examined to determine if its mechanism of inhibition was similar to that of  $\alpha$ -sarcin. Toxin purified to homogeneity from *Shigella dysenteriae* 1, strain 38180, was preincubated with 5  $\mu$ g/ml TPCK-crypsin followed by 19 ng/ml phenylmethylsulfonyl fluoride, 2.5  $\mu$ g/ml urea and 10 mM dithiothreitol. Toxin-inactivation of rabbit reticulocyte ribosomes was carried out by addition of toxin to either lysate or crude ribosomal pellet at a molar ratio of toxin to 80S ribosome of 1:10. [<sup>3</sup>H]leucine incorporation into acid-insoluble material in lysate preparations was completely inhibited by this amount of toxin. Ribosomal RNA was extracted from control or toxin-treated ribosomes and analysed by polyacrylamide-agarose gel electrophoresis. Although fragments derived from 28S rRNA were observed from  $\alpha$ -sarcin-treated ribosomes, no hydrolysis of 28S or 18S rRNA was detected from Shiga toxin-treated ribosomes. Thus, it appears that Shiga toxin inactivates ribosomes in a manner different than  $\alpha$ -sarcin.

Data accumulated to date indicate that Shiga toxin does not hydrolyze rRNA of intact ribosomes to yield a rRNA fragment as does alpha-sarcin, a known 60S ribosomal subunit inactivator and specific RNase. A series of experiments were conducted to determine if shiga toxin possesses RNase activity which could explain how it inactivates 60S ribosomal subunits. Alpha-sarcin was included as a positive control as this fungal-derived toxin is known to specifically hydrolyze rRNA of intact ribosomes to yield an approximate 500 nucleotide fragment from the 3'-terminus of 28S rRNA. Results from one such experiment are presented (Fig. 4). The alpha-sarcin cleavage fragment is designated  $\alpha$ -s. Shiga toxin action did not

result in the formation of  $\alpha$ -s or any other rRNA fragment not present in the control samples. These results were unchanged if ribosomal RNA from control and toxin-treated ribosomes were analyzed on PAGE by silver staining (Fig. 4) or by autoradiography following 3'-end labeling with [5'- $^{32}$ P]pCp and T<sub>4</sub> RNA ligase. Ribonuclease activity of Shiga toxin protein has been observed on free rRNA and is presently being examined more closely.

Fig. 4. Effect of Shiga toxin on rRNA species from toxin-treated reticulocyte ribosomes.



PROCEDURE: RIBOSOMAL SUBUNITS WERE ISOLATED FROM TOXIN-TREATED POLYSOMES (P-100) WITH ANALYSIS OF EXTRACTED rRNA ON A 2.7% ACRYLAMIDE GEL. IN A POLYPHENYLALANINE SYNTHESIS ASSAY, 60S RIBOSOMES TREATED WITH EITHER TOXIN WERE BIOLOGICALLY INACTIVE IN CONTRAST TO 40S RIBOSOMES WHICH WERE NOT AFFECTED BY THE TOXINS.

f. Ribosomal RNAs: Shiga toxin does not result in modification of the 3'-terminal sequence of 5.8S rRNA

A key element of the 60S ribosome is 5.8S rRNA. This component is involved in the ribosomal acceptor site into which aminoacyl-tRNA binds. It also resides in the general location of the lesion resulting from Shiga toxin inactivation of ribosomes. The main conclusion drawn from all of our function data is that Shiga toxin modifies a ribosomal component which is part of the acceptor site. Thus, 5.8S rRNA from Shiga toxin inactivated and control ribosomes was examined more closely. To date, we have sequenced the 3'-terminus of these 5.8S rRNAs. It is concluded that sequence of nucleotides numbered 105 through 158 (the 3'-terminus) are identical in control and toxin-treated samples. The toxin-treated sample is shown in Figure 5. We are presently examining the 5'-terminus of 5.8S rRNA, as well as the 5' and 3'-termini of 5S and 28S rRNA species. This is deemed important because removal of short (1-10NT) stretches from these rRNA species may not have been detected by our previous protocol, but could cause significant reduction in 60S ribosome biological activity.

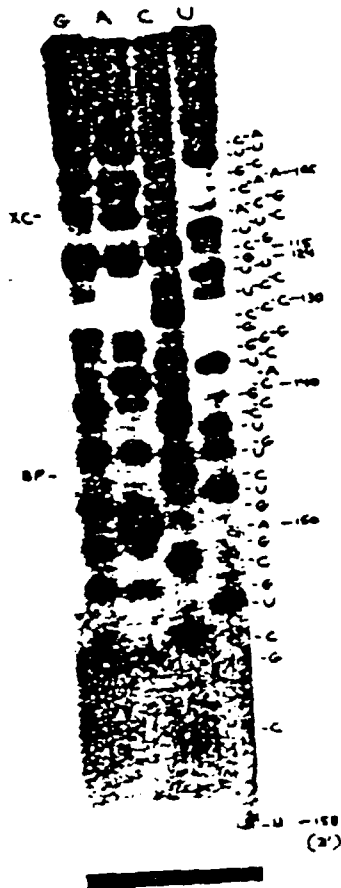


Fig. 5. Effect of Shiga toxin on 3'-terminal sequence of 5.8S rRNA from 60S ribosomal subunits of toxin-treated reticulocyte polysomes.

## Discussion of Results

### a. Initiation of Protein Synthesis

It seems quite clear from the data obtained on this part of the project that Shiga toxin is not a primary inhibitor of the initiation process. Thus, Shiga toxin is without effect on each of the individual steps of initiation as depicted in Scheme "A". This being the case, a working hypothesis has evolved which implicates Shiga toxin as strictly being a ribosome inactivator. Structural modification of the 60S ribosomal subunit most likely results in a single major functional change, namely inhibition of aminoacyl-tRNA binding to ribosomes, a process which requires elongation factor 1. In simple terms, this explains why Shiga toxin inhibits elongation rather than initiation of peptides.

### b. Elongation of Protein Synthesis

As described above, Shiga toxin modifies 60S ribosomes by an enzymatic process to cause a functional lesion in the EF-1-dependent elongation step. The basic question remains as to why EF-1 does not react fully with toxin-treated ribosomes. It is believed that the answer to this will come only when the structural modification in ribosomes is identified.

Our most recent data indicate that Shiga toxin does not inhibit EF-2-dependent reactions. Thus, its primary effect is on EF-1-dependent reactions.

### c. Ribosomal Proteins

In order to identify the physical modification Shiga causes in the 60S ribosome one must, at a maximum examine the 47 ribosomal proteins for changes in size or charge. This is most easily accomplished by the technique of 2-D gel electrophoresis as we have performed. Separation of proteins in the 1st dimension is a function of charge. Since most of the r-proteins are basic, they will carry positive charges at pH 8.6, and migrate towards the cathode. However, because some of these r-proteins have isoelectric points between pH 10 and 11 (more basic than histones) it is probable that small changes in charged amino acids on these proteins would go undetected by electrophoresis at pH 8.6. Thus, we have also started to examine these proteins by running the 1st dimension at either pH 9.6 or 10.6. The second dimension separation is based on size exclusion of proteins from the pores of a high percentage polyacrylamide matrix. Smaller proteins run faster through the gel than do larger proteins. Results from this study have not shown reproducible differences between protein patterns from control vs. Shiga toxin-treated ribosomes. However, a complete examination of the r-proteins with the varied conditions is yet to be completed.

### d. Ribosomal RNAs

Of the three rRNA species present in 60S ribosomes, we have been unable to detect a change in their size as a result of Shiga toxin action on ribosomes. The limit of detection for size changes is approximately 10 nucleotides. As a positive control, we have had little difficulty resolving a 480 nucleotide fragment released from 28S rRNA by alpha sarcin toxin action on 60S ribosomes. The data presented in this report, together suggest that Shiga toxin has a mode of action different from that of alpha sarcin.

As mentioned earlier, Shiga toxin may have specific exonuclease activity whereby, 1 to 10 nucleotides are removed from either the 3' or 5' termini of the 5S, 5.8S or 28S rRNAs. This can only be determined by sequencing of the termini of these rRNAs. Initial sequencing data of the 3' terminus of 5.8S rRNA indicates that this Shiga toxin does not have its action at this site. The 5.8S rRNA sequence has been strongly conserved through evolution. Our

sequence data matches very closely to that of yeast and mouse hepatoma 5.8S rRNA and is identical through the first 35 nucleotides starting from the 3' terminus. We are presently in the process of checking the other termini of rRNAs from Shiga toxin-treated 60S ribosomes.

Using this technique we would be unable to detect a difference in these rRNA species should only a few nucleotides be cleaved from their termini by Shiga toxin. Thus, we have started to examine the rRNAs more closely by the Maxam-Gilbert sequencing procedure. The 5.8S rRNA has been isolated from Shiga toxin-treated 60S ribosomes, 3' end-labeled with [ $^{32}\text{P}$ ] using T4 RNA ligase, processed with base-specific chemical hydrolysis and resolved on a 12% polyacrylamide gel. An autoradiograph of this gel is presented in Figure 5. The nucleotide sequence revealed from this pattern indicates that 3' terminus of 5.8S rRNA obtained from Shiga toxin-treated 60S ribosomes is virtually identical to that of published sequences of 5.8S rRNA from other sources. The control 5.8S rRNA has just been sequenced and agrees with this concept.

## Conclusions

The conclusions to be drawn from our experimentation to date are the following:

1. Shiga toxin does not significantly inhibit initiation of eukaryotic protein synthesis.
2. The major functional lesion caused by Shiga toxin action on ribosomes is located at the aminoacyl-tRNA binding step of the peptide elongation process.
3. Shiga toxin does not affect EF-2 reactions in peptide elongation.
4. We have confirmed a very recent report that Shiga toxin specifically inactivated 60S but not 40S ribosomal subunits.
5. Shiga toxin does not cause major electrophoretic of size changes in ribosomal proteins from 60S ribosomes.
6. Both Shiga toxin and alpha sarcin were shown to specifically inhibit the biological activity of 60S ribosomes in protein synthesis.
7. Incubation of ribosomes with an inhibitory concentration of either toxin yielded fragmentation of ribosomal RNA only in ribosomes treated with alpha sarcin.
8. Shiga toxin and alpha sarcin appear to interact with 60S ribosomes in a different manner although both toxins cause a similar functional lesion in aminoacyl-tRNA binding into the ribosomal A-site.
9. The 3'-terminus of 5.8S rRNA from Shiga toxin-inactivated 60S ribosomes is virtually identical to that of control 5.8S rRNAs.

## Recommendations

Several modes of investigation may be pursued which will ultimately reveal, at the biochemical level, the mechanism by which Shiga toxin inactivates eukaryotic ribosomes. These directions of study are the following:

1. Analysis of ribosomal proteins using refined gel electrophoretic techniques as are presently being applied in this project.
2. To complete the sequence analysis of rRNA species of toxin treated vs. control ribosomes.
3. Application of the chemical crosslinking technique to this problem to help identify the topographical location of Shiga toxin binding and modification site on 60S ribosomes. That is, Shiga toxin, labeled with <sup>125</sup>I could be crosslinked to ribosomes using bifunctional chemical reagents and, following dissociation of ribosomes into their protein and RNA components, the moiety to which the toxin is attached would be identified. A line of experimentation by which to accomplish this latter approach is presented in the contract renewal protocol.

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